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**(54) Nucleotide sequence coding for an outer membrane protein from Neisseria meningitidis and use of said protein in vaccine preparations**

Nukleotidsequenz, die für ein Aussenmembran-Protein von Neisseria meningitidis kodiert und Verwendung dieses Proteins zur Herstellung von Impfstoffen

Séquence nucléotidique codant pour une protéine de la membrane externe de Neisseria meningitidis, et utilisation de cette protéine dans la préparation de vaccins

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**EP 0 474 313 B1****Description**

The present invention is in the field of Genetic Engineering and Biotechnology. More in particular, the invention is related to a nucleotide sequence obtained from the pathogenic bacterium *Neisseria meningitidis*, which nucleotide sequence codes for a protein belonging to the outer membrane of said bacterium. Said protein is cloned and expressed in the host *Escherichia coli*. The characteristics of this protein as well as its capacity to induce immunologically active antibodies (bactericidal antibodies) in its natural host, allow its use in vaccine preparations against pathogenic strains of this microorganism.

The gram-negative bacterium *N. meningitidis* is responsible for one of every three cases of bacterial meningitis in the world. It was described for the first time by Anton Weichselbaum in 1887 (I. DeVoe, 1982, Microbiol. Revs. 46: 162-190), and man (i.e. human beings) is its only natural host up to date.

In the first half of this century some essential aspects were found in relation to the metabolism and serological differentiation of this microorganism. The first unsuccessful attempts to obtain vaccine preparations were based on its capsular polysaccharide (E. Kabat et al., 1945, J. Exp. Med. 80: 299-307). According to the chemical composition of this capsular polysaccharide, the bacterium *N. meningitidis* is serogrouped in A, B, C, 29-E, H, I, K, L, W-135, X, Y or Z, and the major percentage of illness is caused by A, C, Y, W-135 and B. Non-encapsulated strains are not associated with the invasive disease.

Using different methods of purification of these polysaccharides (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1349-1365) the four first polysaccharides (PS) showed to be good immunogens and inducers of bactericidal antibodies in humans (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1367-1384). The presence of this kind of antibodies has been correlated previously with non-susceptibility to the infection (I. Goldschneider et al., 1969, J. Exp. Med. 129: 1307-1326). As of today mono-, bi- or tetravalent vaccines have been well studied for serotypes A, C and W-135 (F. Ambrosch et al., 1983, Bulletin of the WHO 61: 317-323; I. Vodopija et al., 1983, Infect. Immunol. 42: 599-604; M. Cadoz et al., 1985, Vaccine 3: 340-342; H. Peltola et al., 1985, Pediatrics 76: 91-96).

These vaccines have been licensed for their use in humans in different countries (Centers for Disease Control, 1985, Morbid. Mortal. Weekly Report 34: 255-259) and some of them are commercially available from different firms and producers (Connaught Laboratories, USA; Smith Kline-RIT, Belgium; Institute Merieux, France; Behringwerke Aktiengesellschaft, Germany; Istituto Sieroterapico e Vaccino genea Toscano "Sclavo", Italy; Swiss Serum and Vaccine Institute, Berne, Switzerland; among others).

However, the conventional vaccine against *N. meningitidis* serogroup C does not induce sufficient levels of bactericidal antibodies in children under 2 years old, which are the principal victims of this disease. It has been demonstrated that the titer of specific antibodies against *N. meningitidis* in children under four years of age, after three years of vaccination, is similar in vaccinated and in non-vaccinated ones (H. Kayhty et al., 1980, J. of Infect. Dis. 142: 861-868). Also, no memory response was found against *N. meningitidis* after 8 years of vaccination in young adults (N. Rautonen et al., 1986, J. of Immunol. 137: 2670-2675).

The polysaccharide corresponding to *N. meningitidis* serogroup B is poorly immunogenic (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1349-1365) and induces a poor response of IgM of low specificity (W. Zollinger et al., 1979, J. Clin. Invest. 63: 836-848). There are different theories related to this problem, such as cross-reactivity between B polysaccharide and fetal brain structures, antigenic structures modified in solution and sensitivity to neuroaminidases (C. Moreno et al., 1985, Infect. Immun. 47: 527-533). Recently, a chemical modification of PS B was achieved, which induced a response in the host (H. Jennings et al., 1988, US Patent 4 727 136; F. Ashton et al., 1989, Microb. Pathogen. 6: 455-458), but safety of this vaccine in humans has not been demonstrated.

Due to the lack of an effective vaccine against *N. meningitidis* B, and because the risk of endemic infection is low and mainly restricted to children, a routine immunization with polysaccharides is not recommended (C. Frasch, 1989, Clin. Microbiol. Revs. 2: S134-S138) except in the case of an epidemic.

Since after the Second World War the disease was caused in most of the cases by *N. meningitidis* B, vaccines against serogroup B gained special significance.

Other outer membrane components of *N. meningitidis* include phospholipids, lipopolysaccharides (LPS or endotoxins), pili proteins and others. Different immunotypes of LPS have been described for *N. meningitidis* (W. Zollinger and R. Mandrell, 1977, Infect. Immun. 18: 424-433; C.M. Tsai et al., 1983, J. Bacteriol. 155: 498-504) and immunogenicity using non-toxic derivatives was assayed (H. Jennings et al., 1984, Infect. Immun. 43: 407-412) but their variability (H. Schneider et al., 1984, Infect. Immun. 45: 544-549) and pyrogenicity (when it is conjugated to lipid A) are limiting factors up to now.

The pili, structures needed to fix cells to nasopharyngeal mucous membrane (D. Stephens et al., 1983, The J. Infect. Dis. 148: 369-376) have antigenic diversity among different strains (J. Greenblatt et al., 1988, Infect. Immun. 56: 2356-2362) with some common epitopes (D. Stephens et al., 1988, The J. Infect. Dis. 158: 332-342). Presently there are some doubts in relation to the effectiveness of a vaccine based on these structures. However some of these types of vaccine have been obtained, without known results related to their use in humans (C. Brinton, 1988, US Patent

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4 769 240).

Recently, the attention has switched to the other proteins of the outer membrane of this bacterium. There are many immunological types of these protein complexes.

The strains of *N. meningitidis* are subdivided in serotypes according to the presence of specific epitopes in the majoritary protein P1/P2 and in subtypes according to other epitopes in protein P1 (C. Frasch et al., 1985, Rev. Infect. Dis. 7: 504-510).

There are several published articles and patent applications concerning vaccines based on cocktails of these proteins, with previous selective removal of endotoxins using biocompatible detergents. The immunogenicity of these cocktails in animals and humans has been demonstrated (W. Zollinger et al., 1979, J. Clin. Invest. 63: 836-848; C. 10 Frasch and M. Peppler, 1982, Infect. Immun. 37: 271-280; E. Beuvery et al., 1983, Infect. Immun. 40: 369-380; E. Rosenqvist et al., 1983, NIPH Annals 6: 139-149; L. Wang and C. Frasch, 1984, Infect. Immun. 46: 408-414; C. Moreno et al., 1985, Infect. Immun. 47: 527-533; E. Wedege and L. Froholm, 1986, Infect. Immun. 51: 571-578; C. Frasch et al., 1988, The J. Infect. Dis. 158: 710-718; M. Lifely and Z. Wang, 1988, Infect. Immun. 56: 3221-3227; J. Poolman et al., 1988, In J. Poolman et al (Eds), Gonococci and Meningococci, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 159-165; E. Rosenqvist et al., 1988, J. Clin. Microbiol. 26: 1543-1548), including results in massive field trials e.g. Capetown, South Africa in 1981 (C. Frasch, 1985, Eur. J. Clin. Microbiol. 4: 533-536); Iquique, Chile, 1987 (W. Zollinger, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center) and Cuba 1986 and 1988 (G. Sierra, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center). However, with the exception of the last case, the bactericidal 15 antibodies induced by these preparations were restricted to the same serotype strains or related ones.

One of these vaccines is referred to in US patent 4,601,903 which is restricted to one of the *Neisseria* types producing meningitis (serotype 2), with a high incidence, but also other serotypes have been isolated with high frequency from patients, such as serotypes 4 (Cuba from 1981 to 1983, H. Abdillahi et al., 1988, Eur. J. Clin. Microbiol. Infect. Dis. 7: 293-296; Finland from 1976 to 1987, H. Kayhty et al., 1989, Scand. J. Infect. Dis. 21: 527-535); 8 (Australia from 1971 to 1980, F. Ashton et al., 1984, Can. J. Med. Biol. 30: 1289-1291) and 15 (Norway from 1982 to 1984, L. 25 Froholm et al., 1985, Proceedings of the Fourth International Symposium on Pathogenic Neisseria. American Society for Microbiology, Chile from 1985 to 1987, S. Ruiz et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center) as well as strains of undefined serotype (F. Ashton et al., 1980, Can. J. Microbiol. 26: 1480-1488; Australia from 1971 to 1980, F. Ashton et al., 1984, Can. J. Med. Biol. 30: 1289-1291; Finland from 1976 to 1987, H. Kayhty et al., 1989, Scand. J. Infect. Dis. 21: 527-535).

The Cuban vaccine achieved in 1988 by the Centro Nacional de Biopreparados (European Patent Application. No. 301 992) has proven to be very effective. It is based on a high molecular weight antigenic complex. It possesses a broad range of cross-reactivity with other strains and produces and maintains bactericidal antibodies in the immunized host.

35 However, the methods employed to obtain this type of vaccine start with the multiplication in an appropriate culture of a microorganism which is highly pathogenic, with the associated biological risk of handling directly the bacteria. Moreover, this kind of preparation contains lipopolysaccharides, a contaminant that, although it may increase the product's effectiveness, shows at the same time undesirable secondary effects because of its powerful pyrogenicity. Also, its variation in minor antigenic components, which form part of the preparation, cannot be controlled in the different batches, which makes it difficult to follow important parameters related to the reactogenicity and immunogenicity.

40 For this reason, there is increasing interest in the identification of nucleotide sequences coding for highly conserved proteins in all strains, and even more so the identification of inducer proteins of bactericidal antibodies common to the majority of pathogenic *Neisseria*, in order to obtain vaccine preparations with a broad spectrum of protection.

There are different proteins with high molecular weight which are present in low amounts in the outer membrane 45 of *N. meningitidis* when this microorganism is grown in conventional culture media but have a strong response in affected individuals (J. Black et al., 1986, Infect. Immun. 54: 710-713; L. Aoun et al., 1988, Ann. Inst. Pasteur/ Microbiol. 139: 203-212) and/or increase their response under special culture conditions (J. van Putten et al., 1987, Antoine van Leeuwenhoek 53: 557-5564; A. Schryvers and L. Morris, 1988, Molecular Microbiol. 2: 281-288 and Infect. Immun. 56: 1144-1149). Some of these proteins are highly conserved among the different strains, in particular those related 50 to the acquisition of iron by the microorganism, that have become interesting vaccine candidates (L. Mocca et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center; C. Frasch, 1989, Clin. Microbiol. Revs. 2: S134-S138).

In addition to pure proteins obtained from the micro-organism or strains of related species (e.g. 37 kD protein, T. 55 Mietzner and S. Morse, 1987, US Patent 4 681 761), several related genes have been cloned and expressed. Among these proteins are the following:

protease IgA1 (J. Koomey and S. Falkow, 1984, Infect. Immun. 43: 101-107);

protein P1 (A. Barlow et al., 1987, Infect. Immun. 55: 2734-2740, and 1989, Molec. Microbiol. 3: 131-139);

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protein P5a (T. Kawula et al., 1988, Infect. Immun. 56: 380-386);  
protein P5c (T. Olyhoek and M. Achtman, 1988, Proceedings of the Sixth International Pathogenic N. Conference. Callaway Gardens Conference Center);  
protein P4 (K. Klugman et al., 1989, Infect. Immun. 57: 2066-2071);  
protein P2 (K. Murakami et al., 1989, Infect. Immun. 57: 2318-2323);

5 and from *N. gonorrhoeae*, which code for proteins with cross-reactivity with their corresponding proteins from *N. meningitidis*:

10 antigen H.8 (W. Black and J.G. Cannon, 1985, Infect. Immun. 47: 322-325);  
macromolecular complex (W. Tsai and C. Wilde, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center);  
15 37 kDa protein, repressed in the presence of iron (S. Berish et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center).

20 The use of these proteins as active vaccine preparation has not been reported or the bactericidal tests of antibodies induced against them were negative, such as in the case of mouse monoclonal antibodies against H.8 (J. Woods et al., 1987, Infect. Immun. 55: 1927-1928).

Up to the moment, the protein P1 located in the outer membrane of *N. meningitidis* is one of the best characterized and studied antigens. This protein presents no variability within the same strain. However, there are more than 17 types of proteins P1 in *Neisseria* which have differences in three variable regions, this being the basis of the classification of *N.* in different subtypes. This protein is very immunogenic in humans (W.D. Zollinger and R.E. Mandrell, 1983, Med. Trop. 43:143-147), eliciting protective antibodies (E. Wedege and L.O. Froholm, 1986, Infect. Immun. 51: 571-578; K. Saukkonen et al., 1987, Microb. Pathogen. 3:261-267), that give it a special importance in vaccine preparations.

25 Some subtypes of proteins P1 have been cloned in *E. coli*, starting from genomic libraries (A.K. Barlow et al., 1989, Molec. Microb. 3:131-139) or using the PCR technique (S. Butcher et al., VIIth International Congress of Neisseria, R. C. Seid, Patent Application WO 90/06696; Brian Mc Guinness et al., 1990, J. Exp. Med. 171:1871-1882, M.C.J. Maiden et al., VIIth International Conference of Neisseria, Berlin, Sept. 9-14, 1990, and 1991, Molec. Microb. 3:727; J. Suker et al., VIIth International Conference of Neisseria, Berlin, Sept. 9-14, 1990). However, up to now, there is no genetic construction able to produce this protein with high levels of expression. Only low levels of expression (D.A. White et al., 1990, Molec. Microb. 4:769-776) or its expression in *Bacillus subtilis* fused to the outer membrane protein A of *E. coli* (omp A) (E. Wahlstrom et al., VIIth International Congress of Neisseria, September 9-14, 1990, Berlin) have been reported.

30 It can be affirmed that up to the moment no antigen has been isolated which is common to all types and serogroups of *N. meningitidis* and is able to produce bactericidal antibodies. For this reason, an antigen of this kind, conjugated or fused to other proteins or polysaccharides of immunological interest, would be relevant as a candidate for bivalent vaccine preparations.

35 40 This invention is related to a nucleotide sequence coding for a protein having a molecular weight of about 64 kilodaltons. This sequence has been found in all *N. meningitidis* serotypes and serogroups tested, as verified by nucleic acid hybridization, Western-blotting, Dot-blot and ELISA.

A technical object of this invention is the identification of a nucleotide sequence which codes for a highly conserved protein and is common to the majority of pathogenic strains of *Neisseria* (named P64k), in order to obtain the protein 45 by a recombinant way with a high grade of purity and in commercially useful quantities, so that it can be employed in diagnostic methods and as an integrating part of a vaccine preparation of broad spectrum of protection.

On the level of genetic information (DNA and RNA), the invention provides a recombinant polynucleotide, comprising a nucleotide sequence coding for a protein P64k of *Neisseria meningitidis*, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1. In a preferred embodiment said nucleotide sequence coding for the 50 protein P64k of *N. meningitidis* essentially consists of the nucleotide sequence shown in SEQ ID NO:1. The recombinant polynucleotide may further comprise a nucleotide sequence of a cloning or expression vector.

The invention also provides a transformed microorganism containing a recombinant polynucleotide as defined above, preferably a transformed microorganism which is capable of expressing the protein P64k of *N. meningitidis*. In a particularly preferred embodiment of the invention, the transformed microorganism is an *Escherichia coli* strain, e.g. *E. coli* strain HB101, transformed with an expression vector containing a nucleotide sequence coding for the protein P64k of *N. meningitidis*, e.g. the expression vector pM-6.

55 The invention also provides a recombinant proteinaceous substance, comprising an amino acid sequence corresponding to the amino acid sequence of a protein P64k of *N. meningitidis*, said protein P64k essentially having the

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amino acid sequence shown in SEQ ID NO:1. Said recombinant proteinaceous substance may essentially consist of protein P64k, or be a fusion protein or a protein/polysaccharide conjugate comprising the amino acid sequence of protein P64k of *N. meningitidis*.

The invention further provides a vaccine composition, comprising a recombinant protein as defined above, together with a suitable carrier, diluent or adjuvant.

In addition, the invention provides a monoclonal antibody, raised against a recombinant proteinaceous substance as defined above and capable of binding a protein P64k of *N. meningitidis*.

The invention also provides a process for preparing a protein P64k of *Neisseria meningitidis*, or a fusion protein comprising protein P64k, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1, comprising the steps of transforming a microorganism with an expression vector containing a nucleotide sequence coding for said protein P64k, or said fusion protein, culturing the transformed microorganism to obtain expression of said protein P64k, or said fusion protein, and isolating said expression product.

One novel aspect of this invention is the gene isolated from the *N. meningitidis* strain B:4:P1.15, which was named M-6 and has as a principal characteristic its stability in *E. coli* vectors. This gene does not produce adverse effects on the host, allowing to obtain yields of over 25 % of total protein (ratio of P64k to total protein from host strain). On the other hand, it has been demonstrated by Southern and Western Blot hybridizations (E. Southern, 1975, J. Mol. Biol. 98: 503-527 and W. Burnette, 1981, Anal. Biochem. 12: 195-203) that protein P64k is present in all the following studied strains of *N. meningitidis*:

- 20 N. meningitidis A
- N. meningitidis B:1
- N. meningitidis B:2
- N. meningitidis B:4
- N. meningitidis B:5
- 25 N. meningitidis B:8
- N. meningitidis B:9
- N. meningitidis B:11
- N. meningitidis B:15
- N. meningitidis B:4:P1.15
- 30 N. meningitidis C
- N. meningitidis B:15:P1.16
- N. meningitidis B:15:P1.16 (H 44/76)
- N. meningitidis B:NT (121/85)
- N. meningitidis B:NT (71/86)
- 35 N. meningitidis B:NT (210/86)

and also in *N. mucosa*, *N. subflava* and *N. gonorrhoeae*.

The protein is not present in *N. cinerea*, *N. lactamica*, *N. sicca* and *N. flavescens*, but these are not of interest because they are not pathogenic.

40 The protein having a molecular weight of about 64 kDa can be localized by electron microscopy in the outer membrane of *N. meningitidis*. Therefore, this antigen is an exposed antigen which is favorable for use in a vaccine preparation. The protein was recognized in Western blot immunoidentification experiments with sera from convalescents and individuals vaccinated with the conventional Cuban vaccine Va-Mengoc-BC (Centro Nacional de Biopreparados, Havana, Cuba). This aspect guarantees the immunogenicity of the antigen and at the same time confirms its presence within the high molecular weight protein fraction constituent of this vaccine, which is responsible of the lasting immune response to the disease.

Another novel aspect is that the protein, which is an object of this invention, produces antibodies with a broad bactericidal spectrum (different serogroups, serotypes and subtypes), a characteristic which has not been reported previously for any protein from *N. meningitidis*.

50 This protein obtained in high levels in *E. coli* becomes an important candidate for the improvement of immunogenicity when expressed as a fusion protein with other proteins. It could also increase the expression by conferring enhanced stability and suitability in the molecular structure during transcription and translation processes. Belonging to *Neisseria*, this protein can also be fused to other proteins from *Neisseria* in order to obtain vaccine preparations against this microorganism with increased immunogenicity. These fusion proteins are also objects of this invention.

55 On the other hand, surprisingly, it was found that the gene M-6 obtained from a genomic library of the strain *N. meningitidis* B:4:P1.15 showed a great homology with sequences of lipoamide-dehydrogenases and acetyl-transferases from other microorganisms and higher organisms.

An important object of this invention is the nucleotide sequence which codes for the M-6 gene (SEQ ID NO:1 of

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the Sequence Listing) whose product is the protein P64k.

This gene was derived from the genome of the strain B385 isolated in Cuba (*N. meningitidis* B:4:P1.15), by the construction of a genomic library in the phage EMBL 3.

The recombinant DNA including the gene M-6 constitutes another object of this invention, which includes the phage lambda, the plasmid pM-3 and the expression vector pM-6 for expression in bacteria.

In particular, for the intracellular expression in *E. coli*, the M-6 gene was cloned under the tryptophane promotor and using its own termination signal of transcription and a linkage fragment between M-6 and the cloning site Ncol which adds the following nucleotide sequence at the 5' end:

10

ATG CTA GAT AAA AGA

(SEQ ID NO:2)

15 The N-terminal of the protein P64k encoded by the M-6 gene inserted in plasmid pM-6 which adds 5 aminoacids to the N-terminal of the original protein corresponds to:

20

M L D K R M A L V E L K V P D I G G H E N V D I I  
(SEQ ID NO:3)

Another object of this invention are the microorganisms resulting from the transformation of *E. coli* strain HB 101 with the pM-6 vector, which are characterized by the expression of high levels of protein P64k, good viability and great stability.

25 The transformed clone of *E. coli* was denominated HBM64 (Fig. 2), and presents levels of expression of P64k higher than 25 % in relation to the total protein of the cell (Fig. 6).

The procedure described in the present invention, due to the levels of expression achieved for this product, allows to reach an optimal purity for use of this protein in humans.

On the other hand, the antigen obtained from the isolated sequence was very useful in the preparation of different 30 types of potential vaccine preparations, like bivalent vaccines with a broad immunoprotective spectrum, e.g., protein-polysaccharide conjugates, fusion proteins, etc.

EXAMPLES: These examples intend to illustrate the invention, but not to limit the scope of this invention.

35 EXAMPLE 1:

For the isolation of genomic DNA from *N. meningitidis* B:4:P1.15, the cells were grown in Mueller-Hinton medium (OXOID, London). The biomass from a culture of 100 ml was resuspended in 8 ml of Tris (hydroxymethyl-aminomethane) 100 mM, EDTA (ethylenediamine tetraacetic acid) 1mM, pH 8. The cells were subjected to a treatment with lysozyme (10 mg/ml), followed by 200 µl of self-digested pronase (20 mg/ml) and 1.1 ml of 10% SDS. The mixture was incubated at 37°C during 1 hour, then it was treated with phenolchloroform (v/v) and the remains of phenol were eliminated using 2-butanol. Finally, the DNA was precipitated with absolute ethanol and RNA was eliminated with ribonuclease A (Sigma, London).

The DNA of about 60 kb was subjected to a partial digestion with the enzyme Sau 3A, obtaining a population of 45 fragments of about 15 kb. This majority fraction was isolated and purified by separation in agarose gel (T. Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.: Cold Spring Harbor NY).

For the construction of the genomic library, the process described by Maniatis was essentially followed (T. Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.: Cold Spring Harbor NY). Four µg of purified DNA were ligated with 8 µg of BamHI-digested EMBL-3. The ligation product was packed and the phages were 50 finally plated on the *E. coli* strain C66P2.

The library was screened by immunoidentification (R. Young and R. Davis, 1983, PNAS USA 80: 1194-1198) using rabbit serum obtained against a preparation of proteins belonging to the outer membrane of the strain *N. meningitidis* B:4:P1.15. The clones were analyzed by Western-blot (Burnette, 1981) and the expression of the P64k protein with a molecular weight of about 70 kDa was detected. The resulting recombinant phage was named 31. The Western blot 55 was also made using a mixture of sera from convalescents of meningococcemia, free of antibodies from *E. coli*, obtaining the same result as that using hyperimmunized rabbit sera.

This experiment was repeated using sera from several healthy individuals, and the signal obtained was negative against the recombinant protein P64k.

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## EXAMPLE 2:

For subcloning in bacteria, the 17 kb insert corresponding to the phage isolated from the library was cloned in the plasmid pUC18 after separation from the phage's arms using the enzyme Sall. This resulted in the construction pM-1 (Fig. 2), that was subjected to restriction analysis (Fig. 3).

The fragment Sall-HindIII of about 6 kb was recloned in the plasmid pUC18 and the construction pM-2 was obtained (Fig. 2). In order to obtain a more exact localization of the gene coding for protein P64k deletions were carried out with the enzymes Clal, EcoRI and HindII. The complete fragment of the gene M-6 was finally localized as an EcoRI-HindIII insert corresponding to the construction pM-3 (Fig. 2).

In all constructions, the presence of the gene was confirmed by recognizing the protein by colony immunoidentification and Western Blot using hyperimmunized rabbit sera.

The sequence of the insert in pM-3 was determined by the method of Sanger (F. Sanger et al., 1977, PNAS USA 74: 5463-5467).

From the obtained sequence, the approximate molecular weight of the protein encoded by the gene was deduced.

In order to obtain a construction for high expression of the protein P64k, the plasmid pM-3 (Fig. 2) was linearized with the enzyme EcoRI and successive suppressions of the gene were carried out, incubating the sample with the nucleases ExoIII and S1.

The resulting fragments were separated from the rest of the vector pUC18 by cutting with the restriction enzyme HindIII and were cloned fused to a stabilizer fragment (European patent application EP-A-0 416 673), using an Xba-blunt adapter to conserve the XbaI site of the stabilizer gene:

5' C T A G A T A A A A G A 3' (SEQ ID NO:4)

25

3' T A T T T T C T 5' (SEQ ID NO:5)

30 The constructions in which the fused fragment coincided with the reading frame were selected by immunoidentification using hyperimmune rabbit sera.

The insert sequences were established using Sanger's Method (F. Sanger et al., 1977, PNAS USA 74: 5463-5467). From the obtained sequences the approximate molecular weight of the protein encoded by this gene was deduced.

35 The fusion region between the proteins was localized in the gene sequences. In the clone pILM-25 (Figure 4) the ATG of the gene predetermined by the sequence of the DNA insert isolated from the library, coincided with the fusion site.

The Ncol-XbaI fragment, corresponding to the stabilizing peptide coding sequence, was deleted from pILM-25, obtaining a non-fused protein expressed under the tryptophan promoter with its original terminator from the N. meningitidis B:4:P1.15, according to the pM-6 construction (Figure 1).

40 The pM-6 plasmid was transformed in different strains of E. coli like W3110, JA-221, HB-101, LE-392 and MC-1061, and the expression of P64k was compared. The best results were obtained in W3110, JA-221 and HB-101. These strains were chosen to scale up fermentation, and expression levels up to 25 % of total cell proteins were obtained.

45 EXAMPLE 3:

To confirm the correct expression of the cloned gene the N-terminal region of the intact protein was subjected to the Edman degradation method (P. Edman, 1950, Acta Chem. Scand. 4: 283-293). This technique elucidates the sequence (primary structure) of this region in the molecule.

50 The P64k protein was desalted by gel filtration chromatography (PD-10, Pharmacia), eluted with water and monitored at 280 nm. The protein fraction was concentrated to 0.5 nM/μl. One μl of this solution was applied to a PVDF (polyvinylidene difluoride, Millipore) filter, previously activated with methanol.

The Edman degradation was made using the Knauer's Automatic Sequencer, model 810, connected to a HPLC (High Performance Liquid Chromatography) system, so as to detect the phenylthiohydantoin derivatives of the aminoacids (PTH-aminoacids). The standard procedure of sequencing as recommended by the manufacturer of the equipment was followed. The separation of the PTH-aminoacids was performed in a reverse phase column C-18 (5 μm), 250 mm x 2 mm (Merck), eluted with an acetonitrile gradient (B buffer) in sodium acetate (A buffer), prepared according to the manufacturers, with a 200 μl/minute flow and at 42°C. The PTH-aminoacids were detected at 269 nm.

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Data processing and registration were made in a Shimadzu model CR-6a automatic integrator, using a program for data processing by subtraction of two consecutive chromatograms, to facilitate the evaluation of the Edman degradation cycles. Sequence identification is obtained by the chromatographic evaluation of the corresponding analyzed cycle and confirmed by the chromatogram obtained by subtraction, allowing to determine 25 residues.

5

**EXAMPLE 4:**

To demonstrate that the protein P64k is recognized by the sera of individuals vaccinated with the Cuban Vá-Mengoc-BC preparation (Centro Nacional de Biopreparados, Havana, Cuba.), a Western-Blot was made, with a mixture of 12 sera from adults (immunized with two doses of the Cuban vaccine) diluted in a solution containing defatted milk (Oxoid, London). The experiment included:

10

recombinant protein P64k, purified from *E. coli* HB-101 transformed with the pM-6 plasmid;  
supernatant of the ultrasonic cell rupture of untransformed *E. coli* HB-101;

15

the reaction was revealed with a protein A-colloidal gold conjugate.

It was shown that the protein P64k is recognized by the pool of sera.

20

The bactericidal test against B385 (B:4:P1.15) was made according to the procedure described by Lerrick et al. (Scand. J. Immunol. 32, 1990, 121-128) with modifications. With this objective, a mixture was made of a) a suspension of bacteria, cultured under special conditions (1-5 colony forming units/1), b) Gey's balanced salt solution, c) rabbit sera (3 to 4 weeks) as a source of complement and d) pooled sera from mice, immunized against protein P64k in Aluminium Hydroxyde Gel, and inactivated at 56°C for 30 minutes. The immunization of mice was carried out according to an immunization scheme of 3 doses of 20 µg each. The proportions used in the aforementioned mixture were 1:2:1:1 in a total volume of 125 µl. The mixture was incubated at 37°C during 1 hour and plated in fresh Mueller Hinton Agar (Oxoid, London) supplemented with 5 % calf serum (CubaVet, Habana). The counting of surviving colonies was done after 18 hours of incubation of the plates in an atmosphere of 5 % CO<sub>2</sub> at 37°C.

25

The bactericidal titer was considered as the maximum serum dilution necessary to render a 50 % inhibition of bacterial growth, with respect to the same mixture without the test serum. It was found that 1:20 serum dilution still maintains its bactericidal activity. As negative controls (non bactericidal at 1:2 dilution) pooled sera from mice immunized with Aluminum Hydroxyde Gel, and pooled sera from mice immunized with cuban Hepatitis B recombinant vaccine, were used. The bactericidal effect was specific to the anti-P64k antibodies.

30

**EXAMPLE 5:**

The bactericidal test against different strains of *N. meningitidis* was made using:

35

- 40 1. An ammonium sulphate precipitate of the supernatant harvested from a culture of hybridoma cells secreting monoclonal antibodies specific against P64k (anti P64k)/Sample to analyze.
2. An ammonium sulphate precipitate of the supernatant harvested from hybridoma cells secreting monoclonal antibodies specific against the P1.15 protein present in *N. meningitidis* strain B385 (anti P1.15)/Positive control of the system.

45

The maximum dilutions tested were always 1:16. The maximum dilutions tested which had a bactericidal effect, according to the EXAMPLE 5, are indicated:

50

Strain	anti-P64k	anti-P1.15
B385	1:16	1:16
B:4:P1.15	1:16	1:16
B:14:P1.7	1:16	-
B:NT:NT	1:16	-
B:15:P1.15	1:8	-
B:15:P1.16	1:8	-
B:13	1:8	-

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(continued)

5

Strain	anti-P64k	anti-P1.15
C	1:16	-
A	1:16	-

As seen, the anti-P64k monoclonal antibodies have significant bactericidal titers against different serogroups (A, B and C), serotypes (4, 14, 13, 15 and NT) and subtypes (7, 15, 16 and NT) of bacteria.

10 EXAMPLE 7:

Fusion protein M-14 (P64k and P1.15)

15 In order to obtain a genetic construction for high expression that contained the variable epitopes of the P1.15 protein (Outer membrane protein from *N. meningitidis* B:4:P1.15) fused to the P64k protein, the gene coding for P1.15 protein was cloned using the Polymerase Chain Reaction (PCR). The following region containing the variable immunodeterminants of P1.15:

20

L Q L T E P P S K S Q P Q V K V T K A K S R I R T K I S D F G  
 S F I G F K G S E D L G E G L K A V W Q L E Q D V S V A G G G  
 25 A T Q W G N R E S F V G L A G E F G T L R A G R V A N Q F D D  
 A S Q A I D P W D S N N D V A S Q L G I F K R H D D M P V S V  
 R Y D S P D F S G F S G S V Q F V P I Q N S K S A Y T P A Y H  
 Y T R Q N N A D V F V P A V V G K P G S D V Y V A G L N Y K N  
 30 G G F A G S Y A F K Y A R H A N V G R N A F E L F L L G S T S  
 D E A

(SEQ ID NO:6)

35 was inserted in the Mlu I site of the gene M-6, encoding for P64k, after having been made blunt with the klenow fragment from DNA polymerase I. The sites for gene fusion of P1.15 with M-6 are the following:

GDALQL

(SEQ ID NO:7)

40

Gly Asp Ala Leu Gln Leu  
 5' - GGC GAC GCG CTG CAG TTGA -3' (SEQ ID NO:8)  
 45 M-6 P1.15

50

EANAYE

(SEQ ID NO:9)

55

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Glu Ala Asn<sup>\*</sup>Ala Tyr Glu  
5' - GAA GCC AAC GCG TAC GAA -3' (SEQ ID NO:10)  
P1.15 M-6

<sup>\*</sup>: N does not belong to any of the fusion proteins and was created by the genetic construction.

The resulting fusion protein (M-14) was expressed in E. coli using a plasmid vector under the tryptophan promoter, to levels higher than 10 % of total cell protein. The protein was recognized by bactericidal monoclonal antibodies, and anti-P1.15 and P64k polyclonal antibodies, in Western-Blot.

EXAMPLE 8:

15 Polysaccharide/P64k conjugation.

The protein P64k was conjugated with the polysaccharide from Haemophilus influenzae using the reductive amination method. The Haemophilus influenzae polysaccharide (Polyribosyl ribitol phosphate, PRP) was purified by the cold phenol method described by Frasch, 1990 (in: Bacterial Vaccines, 1990, Alan R. Liss, Inc., pp. 123-145). The final contamination of PRP with proteins or nucleic acids was less than 1 %. This polysaccharide was degraded using the method of Parikh et al. 1974 (Methods in Enzymol. 34B: 77-102) with sodium periodate in PRP (ratio 1 : 5 w/w) dissolved in 0.1 M sodium acetate (pH 4.5). The incubation was carried out in the dark during 30 minutes with stirring. The periodate excess was eliminated by addition of ribitol. Very low molecular weight compounds were eliminated by dialysis (Medicell International Ltd. Membrane, London). The resulting oligosaccharide had free aldehyde groups able to react with primary amines (e.g. lysine residues in proteins). The conjugate is obtained by mixing protein and polysaccharide in a 1 : 1 ratio (w/w), adding sodium cyanoborohydride and subjecting the mixture to an incubation, first for 48 hours at 4°C and later at 37°C for 24 hours. The high molecular weight complex which contains the resulting conjugate with protein-polysaccharide in a 1 : 2.3 ratio, can be separated from the non reactive contaminants by HPLC.

30 EXAMPLE 9:

Bivalent vaccine preparation against Hepatitis B virus and N. meningitidis.

In order to obtain a bivalent vaccine preparation, different quantities of protein P64k and Hepatitis B Surface Antigen (Vacuna Recombinante contra la Hepatitis B, Heber Biotec, Havana, Cuba) were mixed. The antigens were adjuvanted with Aluminum Hydroxyde Gel, at 2mg/dose and inoculated in Balb/c mice having a body weight of 20 g in 3 dosis of 0.5 ml each. Different variants were assayed:

- 40 1. P64k 20 µg (P20)
2. HBsAg 20 µg (H20)
3. P64k 10 µg + HBsAg 10 µg (P10:H10)
4. P64K 15 µg + HBsAg 5 µg (P15:H5)
5. Placebo (Al(OH)<sub>3</sub>)

45 Seven days after the immunization with the first doses, the second doses were applied. The third dose was given 14 days after the second. Seven days later, blood was taken and the serum of each immunized animal was separated. Antibody titers against P64k protein were measured in solid phase Enzyme Linked Immunosorbent Assay (ELISA), using P64k at 5 mg/ml to coat the polystyrene plate. The antibody titers against HBsAg were determined by a Commercial ELISA (Organon Teknika, Boxtel). Figure 5 shows the dynamics of antibody response against protein P64k, using sera diluted 1/10 000. The response against P64k is not interfered by the presence of the other antigen. Figure 6 shows the titers against HBsAg after each dosis. The titers against this protein are not diminished by the presence of P64k in the preparation. High titers are obtained against both antigens in the same vaccine preparation.

55 EXAMPLE 10:

A software was created to search the EMBL (European Molecular Biology Laboratory) Data Base and detect the homology between P64k and other proteins. As result of the search it was found that there is homology of one segment in the sequence of P64k with segments in the sequences of N. gonorrhoeae. This sequence was found as characteristic

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in both *N. gonorrhoeae* and *N. meningitidis* (F.F. Correia, S. Inouye and M. Inouye, 1988, J. Biol. Chem. 263, No. 25, 12194-12198).

Another region with high homology was found in two proteins of the Pyruvate Dehydrogenase Complex from *E. coli* K12:

5

a) Acetyltransferase from *E. coli* and the P64k outer membrane protein from *N. meningitidis*.

Homology exists between a segment comprising 100 amino acids, repeated at the beginning of the amino acid sequence of the Acetyltransferase ("Lipoyl Domain", including the "Lipoyl Binding Site" (P.E. Stephens et al., 1983, Eur. J. Biochem. 133, 481-489)) and a region located in the first 111 amino acids of the P64k:

10

MALVELKVPDIGGHENVDIIAVEVNVGDTIAV (SEQ ID NO:11)  
 - \*- \*\*\*\*\* - \*-\* \* \* \*\*\* --  
 VKEVNVPDIGG DEVEVTEVMVKVGDKVAA (SEQ ID NO:12)

20

DDTLITTLEDKATMDVPAEVAGVVKEVKVKVG (SEQ ID NO:11) (cont)  
 -- \*-\*-- \* \* \* - \*-\* \* \* \* \*-\* \* \*  
 EQSLITVEGDKASMEVPAPFAGVVKELKVNVG (SEQ ID NO:12) (cont)

25

DKISEGGLIVVVEAEGT--AAPAKAESAA--A (SEQ ID NO:11) (cont)  
 \*-- \* \*-- \* \* \* \* \* \* - \* \* \*  
 DKVKTGSLIMIFEVEGAAPAAAPAKQEAAAPA (SEQ ID NO:12) (cont)

30

PRKKPLKCRWVPQAAQFGG (SEQ ID NO:11) (cont)  
 \* \* \* \* \*  
 PAAKAEAPAAAPAAKAEGK (SEQ ID NO:12) (cont)

35

where (\*) indicates positions with the same amino acids and (-) indicates positions of conservative amino acid changes.

b) Lipoamide Dehydrogenase from *E. coli* and Outer Membrane P64k protein from *N. meningitidis*.

40

Homology exists between the Lipoamide Dehydrogenase from *E. coli* (a protein having 473 amino acids, P.E. Stephens et al., 1983, Eur. J. Biochem. 133, 481-489) and the protein P64k, specifically in a segment which represents almost the total protein, except the region with homology with the "lipoyl domain" from Acetyltransferase.

45

|---1----|  
 SADAEYDVVVLGGGPGGYSAFAAADEGLKVA (SEQ ID NO:13)  
 \*-- - - \* \* \* - \* \* \* \* \* \* \* \* \* \*  
 STEIKTQVVVLGAGPAGYSAAFRCADLGLETV (SEQ ID NO:14)

50

|-----2-----|  
 IVERYKTLGGVCLNVGCIPSKALLHNAAVIDE (SEQ ID NO:13) (cont)  
 \* \* \* \* - \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* \*  
 IVERYNTLGGVCLNVGCIPSKALLHVAKVIEE (SEQ ID NO:14) (cont)

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5 VRHLAANGIKYPEPALDIDMLRAYKDGVVSRL (SEQ ID NO:13) (cont)  
-- \*---\* - \*\* \*\*\*---\*---\*---\*  
AKALAEHGVGEPKTDIDKI TWKEKVINQL (SEQ ID NO:14) (cont)

10 TG-FGRYGEKRKV DVIQGDGQFLDPHHLEVSL (SEQ ID NO:13) (cont)  
\*\* -- -- \*\*\*---\* ---\* ---\* ---\* ---\*  
TGGLAGMAKGRKV KV VNGL GKFTGANTLEVEG (SEQ ID NO:14) (cont)

15 TAGDAYEQAAAPTGEKKIVAFKN CIIAAGSRVT (SEQ ID NO:13) (cont)  
---\* -----\* ---\* -----\* ---\* -----\*  
ENG-----KTVINF DN AIIAAGSRPI (SEQ ID NO:14) (cont)

20

25 KLPFIP-EDPRIIDSS GAL ALKEVPGKLLIIG (SEQ ID NO:13) (cont)  
-\*\*\*\*\* \* \*\*\*\*\* \*-----\*-----\*-----\*  
QLPFIP HEDPRIWDST DALE LKEVPER LLVMG (SEQ ID NO:14) (cont)

30 GGIIGLEMGTVY STLGSRL DV VEMMDGL MQGA (SEQ ID NO:13) (cont)  
\*\*\*\*\* -----\* -----\* -----\* -----\*  
GGIIGLEMGTVY HAL GSQID VVEMFDQVIPAA (SEQ ID NO:14) (cont)

35

40 DRDLVKVWQKQNEYRFDNIMVN TKTVAVEPKE (SEQ ID NO:13) (cont)  
\*-----\* -----\* -----\* -----\* -----\*  
DKDIVKVFTKRISKKF N-LMLETKVTAVEAKE (SEQ ID NO:14) (cont)

45 DGYYVT FEGANPPKEPQR YDAVLVAAGRAPNG (SEQ ID NO:13) (cont)  
\*-----\* -----\* -----\* -----\* -----\*  
DGIVYVT MEGKKAPAE PQR YDAVLVAIGRVPNG (SEQ ID NO:14) (cont)

50 KLISAEKAGVAVTDRGFIEVDKQMRTNVPHIY (SEQ ID NO:13) (cont)  
\*-----\*-----\*-----\*-----\*-----\*-----\*  
KNLDAGKAGVEVDDRGFIRVDKQLRTNVPHIF (SEQ ID NO:14) (cont)

55

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5	AIGDIVGQPMLAHKAVHEGHVAAENCAGTKAY ***** AIGDIVGQPMLAHKGVHEGHVAAEVIAGKKHY	(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
10	FDAAVIPGVAYTSPEVAWVGETELSAKRPAKG ***- ***--***-***** ** -** - - FDPKVIPSIAYTEPEVAWVGLTEKEAKEKGIS	(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
15	ITKANFPWAASGRAIANGCDKPFTKLIFDAET *-*****-**- - - - * - * - * - * - YETATFPWAASGRAIASDCADGMTKLIFDKES	(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
20	GRIIGGGIVGPNGGDMIAKSALPSKLGCDAA *-***-***-***----- -*- - * - * - * - HRVIGGAIVGTVNGGELLGEIGLAIEMGCDAAED	(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
25	    --3-   VGKTIHPRPTLGESIGMAAEVALGTCTDLPPQ -- ***-***-***- * - * - * - * - IALTIHAHPTLHESVGLAAEVFEGSITDLPNP	
30		(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
35	--KKK - MEN1pd *** KAKKK - EC1pd	(SEQ ID NO:13) (cont) (SEQ ID NO:14) (cont)
40		

Where:

- 45 I-1-I: Adenine binding site (FAD)
- I-2-I: Redox active disulphide region
- I-3-I: Active site histidine

Strain deposits:

50 An E. coli HB-101 clone containing the plasmid pM-3 (a pUC18 plasmid containing the 4.1 kb DNA fragment from Neisseria meninigitidis, strain B:4:P1.15, cloned between the EcoRI and HindIII restriction sites), was deposited on August 30, 1991, with the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and obtained deposit number CBS 485 .91.

55 SEQUENCE LISTING

SEQ ID NO:1

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SEQUENCE TYPE : Nucleotide with corresponding amino acid

SEQUENCE LENGTH: 1830 bp

STRANDEDNESS: Single

TOPOLOGY: Linear

5 MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE ORGANISM: N. meningitidis group B

IMMEDIATE EXPERIMENTAL SOURCE: Strain B:4:P1:15 isolated in Cuba

FEATURES: From 1 to 1830 bp mature protein

PROPERTIES: Gene coding for P64k protein from outer membrane of N. meningitidis

10

ATG CTA GAT AAA AGA ATG GCT TTA GTT GAA TTG AAA GTG CCC Met Leu Asp Lys Arg Met Ala Leu Val Glu Leu Lys Val Pro	42
1                                5                                10	

15

GAC ATT GGC GGA CAC GAA AAT GTA GAT ATT ATC GCG GTT GAA Asp Ile Gly Gly His Glu Asn Val Asp Ile Ile Ala Val Glu	84
15                                20                                25	

20

GTA AAC GTG GGC GAC ACT ATT GCT GTG GAC GAT ACC CTG ATT Val Asn Val Gly Asp Thr Ile Ala Val Asp Asp Thr Leu Ile	126
30                                35                                40	

25

ACT TTG GAA ACC GAT AAA GCG ACT ATG GAC GTA CCT GCT GAA Thr Leu Glu Thr Asp Lys Ala Thr Met Asp Val Pro Ala Glu	168
45                                50                                55	

30

GTT GCA GGC GTA GTC AAA GAA GTT AAA GTT AAA GTC GGC GAC Val Ala Gly Val Val Lys Glu Val Lys Val Lys Val Gly Asp	210
60                                65                                70	

35

AAA ATC TCT GAA GGT GGT TTG ATT GTC GTC GTT GAA GCT GAA Lys Ile Ser Glu Gly Leu Ile Val Val Glu Ala Glu	252
75                                80	

40

GGC ACG GCA GCC GCT CCT AAA GCC GAA TCG GCT GCC GCC CCG Gly Thr Ala Ala Ala Pro Lys Ala Glu Ser Ala Ala Ala Pro	294
85                                90                                95	

45

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	CGC AAG AAG CCC CTA AAC GTG CCG CTC CCT GCT CCG CAA GCC	336
5	Arg Lys Lys Pro Leu Asn Val Pro Leu Pro Ala Pro Gln Ala	
	100 105 110	
	GCG CAA TTC GGC GGT TCT GCC GAT GCC GAG TAC GAT GTG GTC	378
	Ala Gln Phe Gly Gly Ser Ala Asp Ala Glu Tyr Asp Val Val	
	115 120 125	
10	GTA TTG GGT GGC GGT CCC GGC GGT TAC TCC GCT GCA TTT GCC	420
	Val Leu Gly Gly Pro Gly Gly Tyr Ser Ala Ala Phe Ala	
	130 135 140	
15	GCT GCC GAT GAA GGC TTG AAA GTC GCC ATC GTC GAA CGT TAC	462
	Ala Ala Asp Glu Gly Leu Lys Val Ala Ile Val Glu Arg Tyr	
	145 150	
20	AAA ACT TTG GGC GGC GTT TGC CTG AAC GTC GGC TGT ATC CCT	504
	Lys Thr Leu Gly Gly Val Cys Leu Asn Val Gly Cys Ile Pro	
	155 160 165	
25	TCC AAA GCC TTG TTG CAC AAT GCC GCC GTT ATC GAC GAA GTG	546
	Ser Lys Ala Leu Leu His Asn Ala Ala Val Ile Asp Glu Val	
	170 175 180	
30	CGC CAC TTG GCT GCC AAC GGT ATC AAA TAC CCC GAG CCG GAA	588
	Arg His Leu Ala Ala Asn Gly Ile Lys Tyr Pro Glu Pro Glu	
	185 190 195	
35	CTC GAC ATC GAT ATG CTT CGC GCC TAC AAA GAC GGC GTA GTT	630
	Leu Asp Ile Asp Met Leu Arg Ala Tyr Lys Asp Gly Val Val	
	200 205 210	
40	TCC CGC CTC ACG GGC GGT TTG GCA GGT ATG GCG AAA AGC CGT	672
	Ser Arg Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Ser Arg	
	215 220	
45	AAA GTG GAC GTT ATC CAA GGC GAC GGG CAA TTC TTA GAT CCG	714
	Lys Val Asp Val Ile Gln Gly Asp Gly Gln Phe Leu Asp Pro	
	225 230 235	
50	CAC CAC TTG GAA GTG TCG CTG ACT GCC GGC GAC GCG TAC GAA	756
	His His Leu Glu Val Ser Leu Thr Ala Gly Asp Ala Tyr Glu	
	240 245 250	
55	CAG GCA GCC CCT ACC GGC GAG AAA AAA ATC GTT GCC TTC AAA	798
	Gln Ala Ala Pro Thr Gly Glu Lys Lys Ile Val Ala Phe Lys	
	255 260 265	
	AAC TGT ATC ATT GCA GCA GGC AGC CGC GTA ACC AAA CTG CCT	840
	Asn Cys Ile Ile Ala Ala Gly Ser Arg Val Thr Lys Leu Pro	
	270 275 280	

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	TTC ATT CCT GAA GAT CCG CGC ATC ATC GAT TCC AGC GGC GCA	882
5	Phe Ile Pro Glu Asp Pro Arg Ile Ile Asp Ser Ser Gly Ala	
	285 290	
	TTG GCT CTG AAA GAA GTA CCG GGC AAA CTG CTG ATT ATC GGC	924
	Leu Ala Leu Lys Glu Val Pro Gly Lys Leu Leu Ile Ile Gly	
	295 300 305	
10	GCG GGC ATT ATC GGC CTC GAG ATG GGT ACG GTT TAC AGC ACG	966
	Gly Gly Ile Ile Gly Leu Glu Met Gly Thr Val Tyr Ser Thr	
	310 315 320	
15	CTG GGT TCG CGT TTG GAT GTG GTT GAA ATG ATG GAC GGC CTG	1008
	Leu Gly Ser Arg Leu Asp Val Val Glu Met Met Asp Gly Leu	
	325 330 335	
20	ATG CAA GGC GCA GAC CGC GAT TTG GTA AAA GTA TGG CAA AAA	1050
	Met Gln Gly Ala Asp Arg Asp Leu Val Lys Val Trp Gln Lys	
	340 345 350	
25	CAA AAC GAA TAC CGT TTT GAC AAC ATT ATG GTC AAC ACC AAA	1092
	Gln Asn Glu Tyr Arg Phe Asp Asn Ile Met Val Asn Thr Lys	
	355 360	
	ACC GTT GCA GTT GAG CCG AAA GAA GAC GGC GTT TAC GTT ACC	1134
	Thr Val Ala Val Glu Pro Lys Glu Asp Gly Val Tyr Val Thr	
	365 370 375	
30	TTT GAA GGC GCG AAC GCC CCT AAA GAG CCG CAA CGC TAC GAT	1176
	Phe Glu Gly Ala Asn Ala Pro Lys Glu Pro Gln Arg Tyr Asp	
	380 385 390	
35	GCC GTA TTG GTT GCC GCC CGC GCG CCC AAC GGC AAA CTC	1218
	Ala Val Leu Val Ala Ala Gly Arg Ala Pro Asn Gly Lys Leu	
	395 400 405	
40	ATC AGC GCG GAA AAA GCA GGC GTT GCC GTA ACC GAT CGC GGC	1260
	Ile Ser Ala Glu Lys Ala Gly Val Ala Val Thr Asp Arg Gly	
	410 415 420	
45	TTC ATC GAA GTG GAC AAA CAA ATG CGT ACC AAT GTG CCG CAC	1302
	Phe Ile Glu Val Asp Lys Gln Met Arg Thr Asn Val Pro His	
	425 430	
	ATC TAC GCC ATC GGC GAC ATC GTC GGT CAG CCG ATG TTG GCG	1344
	Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu Ala	
	435 440 445	
50	CAC AAA GCC GTT CAC GAA GGC CAC GTT GCC GCC GAA AAC TGC	1386
	His Lys Ala Val His Glu Gly His Val Ala Ala Glu Asn Cys	
	450 455 460	

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GCC GGC AAC AAA GCC TAC TTC GAC GCA CGG GTG ATT CCG GGC	1428
Ala Gly Asn Lys Ala Tyr Phe Asp Ala Arg Val Ile Pro Gly	
465 470 475	
5	
GTT GCC TAC ACT TCC CCC GAA GTG GCG TGG GTG GGC GAA ACC	1470
Val Ala Tyr Thr Ser Pro Glu Val Ala Trp Val Gly Glu Thr	
480 485 490	
10 GAA CTG TCC GCC AAA GCC TCC GCG CGC AAA ATC ACC AAA GCC	1512
Glu Leu Ser Ala Lys Ala Ser Ala Arg Lys Ile Thr Lys Ala	
495 500	
15 AAC TTC CCG TGG GCG GCT TCC GGC CGT GCG ATT GCC AAC GGT	1554
Asn Phe Pro Trp Ala Ala Ser Gly Arg Ala Ile Ala Asn Gly	
505 510 515	
20 TGC GAC AAG CCG TTT ACC AAG CTG ATT TTT GAT GCC GAA ACC	1596
Cys Asp Lys Pro Phe Thr Lys Leu Ile Phe Asp Ala Glu Thr	
520 525 530	
25 GGC CGC ATC ATC GGC GGC ATT GTC GGT CCG AAC GGT GGC	1638
Gly Arg Ile Ile Gly Gly Ile Val Gly Pro Asn Gly Gly	
535 540 545	
30 GAT ATG ATC GCG AAG TCT GCC TTG CCA TCG AAA TGG GCT GCG	1680
Asp Met Ile Ala Lys Ser Ala Leu Pro Ser Lys Trp Ala Ala	
550 555 560	
35 ACA CGT GCA GAC ATC GGC AAA ACC ATC CAC CCG CGC CCG ACC	1722
Thr Arg Ala Asp Ile Gly Lys Thr Ile His Pro Arg Pro Thr	
565 570	
40 TTG GGC GAA TCC ATC GGT ATG GCG GCG GAA GTG GCA TTG GGT	1764
Leu Gly Glu Ser Ile Gly Met Ala Ala Glu Val Ala Leu Gly	
575 580 585	
ACT TGT ACC GAC CTG CCT CCG CAA AAG AAA AAA TAA	1800
45 Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys Lys *	
590 595 599	
45 ATCC GACTGAATAA ACAGCCGATA AGGT TTATTT GA	1836

45

SEQ ID NO: 2

SEQUENCE TYPE : Nucleotide  
 SEQUENCE LENGTH: 15 bases

50

ATGCTAGATA AAAGA

15

55

SEQ ID NO: 3

SEQUENCE TYPE : Amino acid  
 SEQUENCE LENGTH: 25 amino acids

**EP 0 474 313 B1**MOLECULE TYPE: Sequence N-terminal of P64k protein from outer membrane of *N. meningitidis*

5 Met Leu Asp Lys Arg Met Ala Leu Val Glu Leu Lys Val Pro Asp  
1 5 10 15

Ile Gly Gly His Glu Asn Val Asp Ile Ile  
20 25

10

SEQ ID NO: 4

SEQUENCE TYPE : Nucleotide  
15 SEQUENCE LENGTH: 12 bases

CTAGATAAAA GA

12

20

SEQ ID NO: 5

SEQUENCE TYPE : Nucleotide  
SEQUENCE LENGTH: 8 bases

25

TCTTTTAT

8

SEQ ID NO: 6

30

SEQUENCE TYPE : Amino acid  
SEQUENCE LENGTH: 221 amino acids  
MOLECULE TYPE: Sequence which includes variable regions of P1.15 protein

35

Leu Gln Leu Thr Glu Pro Pro Ser Lys Ser Gln Pro Gln Val Lys  
1 5 10 15

40

Val Thr Lys Ala Lys Ser Arg Ile Arg Thr Gln Ile Ser Asp Phe  
20 25 30

Gly Ser Phe Ile Gly Phe Lys Gly Ser Glu Asp Leu Gly Glu Gly  
35 40 45

45

Leu Lys Ala Val Trp Gln Leu Glu Gln Asp Val Ser Val Ala Gly  
50 55 60

50

55

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Gly Gly Ala Thr Gln Trp Gly Asn Arg Glu Ser Phe Val Gly Leu  
 65 70 75

5 Ala Gly Glu Phe Gly Thr Leu Arg Ala Gly Arg Val Ala Asn Gln  
 80 85 90

Phe Asp Asp Ala Ser Gln Ala Ile Asp Pro Trp Asp Ser Asn Asn  
 95 100 105

10 Asp Val Ala Ala Ser Gln Leu Gly Ile Phe Lys Arg His Asp Asp  
 110 115 120

Met Pro Val Ser Val Arg Tyr Asp Ser Pro Asp Phe Ser Gly Phe  
 125 130 135

Ser Gly Ser Val Gln Phe Val Pro Ile Gln Asn Ser Lys Ser Ala  
 140 145 150

20 Tyr Thr Pro Ala Tyr His Tyr Thr Arg Gln Asn Asn Ala Asp Val  
 155 160 165

Phe Val Pro Ala Val Val Gly Lys Pro Gly Ser Asp Val Tyr Val  
 170 175 180

25 Ala Gly Leu Asn Tyr Lys Asn Gly Gly Phe Ala Gly Ser Tyr Ala  
 185 190 195

30 Phe Lys Tyr Ala Arg His Ala Asn Val Gly Arg Asn Ala Phe Glu  
 200 205 210

Leu Phe Leu Leu Gly Ser Thr Ser Asp Glu Ala  
 215 220

35

SEQ ID NO: 7

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 6 amino acids

40

MOLECULE TYPE: Sequence corresponding to the fusion site between N-terminal of P64k and P1.15

Gly Asp Ala Leu Gln Leu  
 1 5

45

SEQ ID NO: 8

SEQUENCE TYPE : Nucleotide

50

SEQUENCE LENGTH: 19 bases

MOLECULE TYPE: Sequence corresponding to the fusion of N-terminal from gene M-6 and from gene P1.15

55

GGCGACGCGC TGCAGTTGA

19

SEQ ID NO: 9

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SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 6 amino acids

MOLECULE TYPE: Sequence corresponding to the fusion site between C-terminal of P64k and P1.15

5

Glu Ala Asn Ala Tyr Glu  
1 5

10

SEQ ID NO: 10

SEQUENCE TYPE : Nucleotide

SEQUENCE LENGTH: 18 bases

MOLECULE TYPE: Sequence corresponding to the fusion of C-terminal from gene M-6 and from gene P1.15

15

GAAGCCAACCG CGTACGAA 18

20

SEQ ID NO: 11

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 111 amino acids

MOLECULE TYPE: p64k N-terminal comprising homology region with "lypol binding site" from E. coli Acetyl transferase

30

Met Ala Leu Val Glu Leu Lys Val Pro Asp Ile Gly Gly His Glu  
1 5 10 15

35

Asn Val Asp Ile Ile Ala Val Glu Val Asn Val Gly Asp Thr Ile  
20 25 30

35

Ala Val Asp Asp Thr Leu Ile Thr Leu Glu Thr Asp Lys Ala Thr  
35 40 45

40

Met Asp Val Pro Ala Glu Val Ala Gly Val Val Lys Glu Val Lys  
50 55 60

45

Val Lys Val Gly Asp Lys Ile Ser Glu Gly Gly Leu Ile Val Val  
65 70 75

50

Val Glu Ala Glu Gly Thr Ala Ala Ala Pro Lys Ala Glu Ser Ala  
80 85 90Ala Ala Pro Arg Lys Lys Pro Leu Lys Cys Arg Trp Val Pro Gln  
95 100 105Ala Ala Gln Phe Gly Gly  
110

55

SEQ ID NO: 12

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 112 amino acids

MOLECULE TYPE: "lypol binding site" from E. coli Acetyltransferase

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Val Lys Glu Val Asn Val Pro Asp Ile Gly Gly Asp Glu Val Glu  
1 5 10 15

5 Val Thr Glu Val Met Val Lys Val Gly Asp Lys Val Ala Ala Glu  
20 25 30

Gln Ser Leu Ile Thr Val Glu Gly Asp Lys Ala Ser Met Glu Val  
10 35 40 45

Pro Ala Pro Phe Ala Gly Val Val Lys Glu Leu Lys Val Asn Val  
50 55 60

15 Gly Asp Lys Val Lys Thr Gly Ser Leu Ile Met Ile Phe Glu Val  
65 70 75

Glu Gly Ala Ala Pro Ala Ala Ala Pro Ala Lys Gln Glu Ala Ala  
80 85 90

20 Ala Pro Ala Pro Ala Ala Lys Ala Glu Ala Pro Ala Ala Pro  
95 100 105

25 Ala Ala Lys Ala Glu Gly Lys  
110

SEQ ID NO: 13

30 SEQUENCE TYPE : Amino acid  
SEQUENCE LENGTH: 481 amino acids  
MOLECULE TYPE: P64k fragment comprising the homology region with Lipoamide Dehydrogenase from E.  
coli

35

40

45

50

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Ser	Ala	Asp	Ala	Glu	Tyr	Asp	Val	Val	Val	Leu	Gly	Gly	Gly	Pro	
1				5					10					15	
5	Gly	Gly	Tyr	Ser	Ala	Ala	Phe	Ala	Ala	Ala	Asp	Glu	Gly	Leu	Lys
					20					25				30	
10	Val	Ala	Ile	Val	Glu	Arg	Tyr	Lys	Thr	Leu	Gly	Gly	Val	Cys	Leu
					35				40					45	
15	Asn	Val	Gly	Cys	Ile	Pro	Ser	Lys	Ala	Leu	Leu	His	Asn	Ala	Ala
					50				55					60	
20	Val	Ile	Asp	Glu	Val	Arg	His	Leu	Ala	Ala	Asn	Gly	Ile	Lys	Tyr
					65				70					75	
25	Pro	Glu	Pro	Ala	Leu	Asp	Ile	Asp	Met	Leu	Arg	Ala	Tyr	Lys	Asp
					80				85					90	
30	Gly	Val	Val	Ser	Arg	Leu	Thr	Gly	Phe	Gly	Arg	Tyr	Gly	Glu	Lys
					95				100					105	
35	Arg	Lys	Val	Asp	Val	Ile	Gln	Gly	Asp	Gly	Gln	Phe	Leu	Asp	Pro
					110				115					120	
40	His	His	Leu	Glu	Val	Ser	Leu	Thr	Ala	Gly	Asp	Ala	Tyr	Glu	Gln
					125				130					135	
45	Ala	Ala	Pro	Thr	Gly	Glu	Lys	Lys	Ile	Val	Ala	Phe	Lys	Asn	Cys
					140				145					150	
50	Ile	Ile	Ala	Ala	Gly	Ser	Arg	Val	Thr	Lys	Leu	Pro	Phe	Ile	Pro
					155				160					165	
55	Glu	Asp	Pro	Arg	Ile	Ile	Asp	Ser	Ser	Gly	Ala	Leu	Ala	Leu	Lys
					170				175					180	
60	Glu	Val	Pro	Gly	Lys	Leu	Leu	Ile	Ile	Gly	Gly	Gly	Ile	Ile	Gly
					185				190					195	
65	Leu	Glu	Met	Gly	Thr	Val	Tyr	Ser	Thr	Leu	Gly	Ser	Arg	Leu	Asp
					200				205					210	
70	Val	Val	Glu	Met	Met	Asp	Gly	Leu	Met	Gln	Gly	Ala	Asp	Arg	Asp
					215				220					225	

50

55

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	Leu Val Lys Val Trp Gln Lys Gln Asn Glu Tyr Arg Phe Asp Asn		
	230	235	240
5	Ile Met Val Asn Thr Lys Thr Val Ala Val Glu Pro Lys Glu Asp		
	245	250	255
10	Gly Val Tyr Val Thr Phe Glu Gly Ala Asn Pro Pro Lys Glu Pro		
	260	265	270
	Gln Arg Tyr Asp Ala Val Leu Val Ala Ala Gly Arg Ala Pro Asn		
	275	280	285
15	Gly Lys Leu Ile Ser Ala Glu Lys Ala Gly Val Ala Val Thr Asp		
	290	295	300
	Arg Gly Phe Ile Glu Val Asp Lys Gln Met Arg Thr Asn Val Pro		
	305	310	315
20	His Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu Ala		
	320	325	330
25	His Lys Ala Val His Glu Gly His Val Ala Ala Glu Asn Cys Ala		
	335	340	345
	Gly Thr Lys Ala Tyr Phe Asp Ala Ala Val Ile Pro Gly Val Ala		
	350	355	360
30	Tyr Thr Ser Pro Glu Val Ala Trp Val Gly Glu Thr Glu Leu Ser		
	365	370	375
	Ala Lys Arg Pro Ala Gly Lys Ile Thr Lys Ala Asn Phe Pro Trp		
	380	385	390
35	Ala Ala Ser Gly Arg Ala Ile Ala Asn Gly Cys Asp Lys Pro Phe		
	395	400	405
40	Thr Lys Leu Ile Phe Asp Ala Glu Thr Gly Arg Ile Ile Gly Gly		
	410	415	420
	Gly Ile Val Gly Pro Asn Gly Gly Asp Met Ile Ala Lys Ser Ala		
	425	430	435
45	Leu Pro Ser Lys Leu Gly Cys Asp Ala Ala Asp Val Gly Lys Thr		
	440	445	450
	Ile His Pro Arg Pro Thr Leu Gly Glu Ser Ile Gly Met Ala Ala		
	455	460	465
50	Glu Val Ala Leu Gly Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys		
	470	475	480
55	Lys		

SEQ ID NO: 14

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SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 472 bases

MOLECULE TYPE: Segment of E. coli Lipoamide Dehydrogenase with homology to P64k protein

5

Ser Thr Glu Ile Lys Thr Gln Val Val Val Leu Gly Ala Gly Pro  
1 5 10 15

10

Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu  
20 25 30

Thr Val Ile Val Glu Arg Tyr Asn Thr Leu Gly Gly Val Cys Leu  
35 40 45

15

Asn Val Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys  
50 55 60

20

Val Ile Glu Glu Ala Lys Ala Leu Ala Glu His Gly Ile Val Phe  
65 70 75

Gly Glu Pro Lys Thr Asp Ile Asp Lys Ile Thr Trp Lys Glu Lys  
80 85 90

25

Val Ile Asn Gln Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Gly  
95 100 105

Arg Lys Val Lys Val Val Asn Gly Leu Gly Lys Phe Thr Gly Ala  
110 115 120

30

Asn Thr Leu Glu Val Glu Gly Glu Asn Gly Lys Thr Val Ile Asn  
125 130 135

35

Phe Asp Asn Ala Ile Ile Ala Ala Gly Ser Arg Pro Ile Gln Leu  
140 145 150

Pro Phe Ile Pro His Glu Asp Pro Arg Ile Trp Asp Ser Thr Asp  
155 160 165

40

Ala Leu Glu Leu Lys Glu Val Pro Glu Arg Leu Leu Val Met Gly  
170 175 180

Gly Gly Ile Ile Gly Leu Glu Met Gly Thr Val Tyr His Ala Leu  
185 190 195

45

Gly Ser Gln Ile Asp Val Val Glu Met Phe Asp Gln Val Ile Pro  
200 205 210

50

Ala Ala Asp Lys Asp Ile Val Lys Val Phe Thr Lys Arg Ile Ser  
215 220 225

55

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	Lys Lys Phe Asn Leu Met Leu Glu Thr Lys Val Thr Ala Val Glu		
	230	235	240
5	Ala Lys Glu Asp Gly Ile Tyr Val Thr Met Glu Gly Lys Lys Ala		
	245	250	255
	Pro Ala Glu Pro Gln Arg Tyr Asp Ala Val Leu Val Ala Ile Gly		
10	260	265	270
	Arg Val Pro Asn Gly Lys Asn Leu Asp Ala Gly Lys Ala Gly Val		
	275	280	285
15	Glu Val Asp Asp Arg Gly Phe Ile Arg Val Asp Lys Gln Leu Arg		
	290	295	300
	Thr Asn Val Pro His Ile Phe Ala Ile Gly Asp Ile Val Gly Gln		
	305	310	315
20	Pro Met Leu Ala His Lys Gly Val His Glu Gly His Val Ala Ala		
	320	325	330
25	Glu Val Ile Ala Gly Lys Lys His Tyr Phe Asp Pro Lys Val Ile		
	335	340	345
	Pro Ser Ile Ala Tyr Thr Glu Pro Glu Val Ala Trp Val Gly Leu		
	350	355	360
30	Thr Glu Lys Glu Ala Lys Glu Lys Gly Ile Ser Tyr Glu Thr Ala		
	365	370	375
	Thr Phe Pro Trp Ala Ala Ser Gly Arg Ala Ile Ala Ser Asp Cys		
35	380	385	390
	Ala Asp Gly Met Thr Lys Leu Ile Phe Asp Lys Glu Ser His Arg		
	395	400	405
40	Val Ile Gly Gly Ala Ile Val Gly Thr Asn Gly Gly Glu Leu Leu		
	410	415	420
	Gly Glu Ile Gly Leu Ala Ile Glu Met Gly Cys Asp Ala Glu Asp		
45	425	430	435
	Ile Ala Leu Thr Ile His Ala His Pro Thr Leu His Glu Ser Val		
	440	445	450
	Gly Leu Ala Ala Glu Val Phe Glu Gly Ser Ile Thr Asp Leu Pro		
50	455	460	465
	Asn Pro Lys Ala Lys Lys Lys		
	470		

**EP 0 474 313 B1****Claims**

1. A recombinant polynucleotide, comprising a nucleotide sequence coding for a protein P64k of *Neisseria meningitidis*, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1.
- 5 2. A recombinant polynucleotide according to claim 1, wherein said nucleotide sequence coding for the protein P64k of *N. meningitidis* essentially consists of the nucleotide sequence shown in SEQ ID NO:1.
- 10 3. A recombinant polynucleotide according to claim 1 or 2, further comprising a nucleotide sequence of a cloning or expression vector.
4. A transformed microorganism, containing a recombinant polynucleotide according to any one of claims 1 to 3.
- 15 5. A transformed microorganism according to claim 4, which is capable of expressing the protein P64k of *N. meningitidis*.
6. A transformed microorganism according to claim 5, which is an *Escherichia coli* strain, transformed with an expression vector containing a nucleotide sequence coding for the protein P64k of *N. meningitidis*.
- 20 7. A recombinant proteinaceous substance, comprising an amino acid sequence corresponding to the amino acid sequence of a protein P64k of *N. meningitidis*, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1.
8. A recombinant proteinaceous substance according to claim 7, which is a fusion protein or a protein/polysaccharide conjugate comprising the amino acid sequence of protein P64k of *N. meningitidis*.
- 25 9. A vaccine composition, comprising a recombinant protein according to claim 7 or 8, together with a suitable carrier, diluent or adjuvant.
- 30 10. A monoclonal antibody, raised against a recombinant proteinaceous substance according to claim 7 or 8 and capable of binding a protein P64k of *N. meningitidis*.
11. A process for preparing a protein P64k of *Neisseria meningitidis*, or a fusion protein comprising protein P64k, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1, comprising the steps of transforming a microorganism with an expression vector containing a nucleotide sequence coding for said protein P64k, or said fusion protein, culturing the transformed microorganism to obtain expression of said protein P64k, or said fusion protein, and isolating said expression product.

**40 Patentansprüche**

1. Rekombinantes Polynukleotid, das eine Nukleotidsequenz umfaßt, die für ein Protein P64k von *Neisseria meningitidis* codiert, wobei das Protein P64k im wesentlichen die in SEQ ID NO:1 gezeigte Aminosäuresequenz aufweist.
- 45 2. Rekombinantes Polynukleotid gemäß Anspruch 1, wobei die Nukleotidsequenz, die für das Protein P64k von *N. meningitidis* codiert, im wesentlichen aus der in SEQ ID NO:1 gezeigten Nukleotidsequenz besteht.
3. Rekombinantes Polynukleotid gemäß Anspruch 1 oder 2, das weiterhin eine Nukleotidsequenz eines Klonierungs- oder Expressionsvektors umfaßt.
- 50 4. Transformierter Mikroorganismus, der ein rekombinantes Polynukleotid gemäß einem der Ansprüche 1 bis 3 enthält.
5. Transformierter Mikroorganismus gemäß Anspruch 4, der das Protein P64k von *N. meningitidis* zu exprimieren vermag.
6. Transformierter Mikroorganismus gemäß Anspruch 5, bei dem es sich um einen *Escherichia-coli*-Stamm handelt, der mit einem Expressionsvektor transformiert ist, der eine Nukleotidsequenz enthält, die für das Protein P64k

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von *N. meningitidis* codiert.

7. Rekombinante Proteinsubstanz, die eine Aminosäuresequenz umfaßt, die der Aminosäuresequenz eines Proteins P64k von *N. meningitidis* entspricht, wobei das Protein P64k im wesentlichen die in SEQ ID NO:1 gezeigte Aminosäuresequenz aufweist.  
5
8. Rekombinante Proteinsubstanz gemäß Anspruch 7, bei der es sich um ein Fusionsprotein oder ein Protein/Poly-  
saccharid-Konjugat handelt, das die Aminosäuresequenz von Protein P64k von *N. meningitidis* umfaßt.  
10
9. Impfstoffzusammensetzung, die ein rekombinantes Protein gemäß Anspruch 7 oder 8 zusammen mit einem ge-  
eigneten Träger, Verdünnungsmittel oder Hilfsstoff umfaßt.  
15
10. Monoklonaler Antikörper, der gegen eine rekombinante Proteinsubstanz gemäß Anspruch 7 oder 8 entwickelt  
wurde und in der Lage ist, ein Protein P64k von *N. meningitidis* zu binden.  
20
11. Verfahren zur Herstellung eines Proteins P64k von *Neisseria meningitidis* oder eines Fusionsproteins, das das  
Protein P64k umfaßt, wobei das Protein P64k im wesentlichen die in SEQ ID NO:1 gezeigte Aminosäuresequenz  
aufweist, umfassend die Schritte des Transformierens eines Mikroorganismus mit einem Expressionsvektor, der  
eine Nucleotidsequenz enthält, die für das Protein P64k oder das Fusionsprotein codiert, des Kultivierens des  
transformierten Mikroorganismus, so daß das Protein P64k oder das Fusionsprotein exprimiert wird, und des Iso-  
lierens des Expressionsprodukts.  
25

**Revendications**

1. Polynucléotide recombiné comprenant une séquence nucléotidique codant une protéine P64k de *Neisseria me-*  
*ningitidis*, ladite protéine P64k ayant essentiellement la séquence d'acides aminés montrée dans SEQ ID NO:1.  
25
2. Polynucléotide recombiné selon la revendication 1, dans lequel ladite séquence nucléotidique codant la protéine  
P64k de *N. meningitidis* consiste essentiellement en la séquence nucléotidique montrée dans SEQ ID NO:1.  
30
3. Polynucléotide recombiné selon la revendication 1 ou 2, comprenant en outre une séquence nucléotidique d'un  
vecteur de clonage ou d'expression.  
35
4. Micro-organisme transformé contenant un polynucléotide recombiné selon l'une quelconque des revendications  
1 à 3.  
40
5. Micro-organisme transformé selon la revendication 4, qui est capable d'exprimer la protéine P64k de *N. meningi-*  
*tidis*.  
45
6. Micro-organisme transformé selon la revendication 5, qui est une souche de *Escherichia coli* transformée avec  
un vecteur d'expression contenant une séquence nucléotidique codant la protéine P64k de *N. meningitidis*.  
50
7. Substance protéique recombinée comprenant une séquence d'acides aminés correspondant à la séquence d'aci-  
des aminés d'une protéine P64k de *N. meningitidis*, ladite protéine P64k ayant essentiellement la séquence d'aci-  
des aminés montrée dans SEQ ID NO:1.  
55
8. Substance protéique recombinée selon la revendication 7 qui est une protéine de fusion ou un conjugué protéine/  
polysaccharide comprenant la séquence d'acides aminés de la protéine P64k de *N. meningitidis*.  
60
9. Composition de vaccin comprenant une protéine recombinée selon la revendication 7 ou 8 ainsi qu'un support,  
diluant ou adjuvant approprié.  
65
10. Anticorps monoclonal dirigé contre une substance protéique recombinée selon la revendication 7 ou 8 et capable  
de se lier à une protéine P64k de *N. meningitidis*.  
70
11. Procédé de préparation d'une protéine P64k de *Neisseria meningitidis* ou d'une protéine de fusion comprenant  
une protéine P64k, ladite protéine P64k ayant essentiellement la séquence d'acides aminés montrée dans SEQ  
75

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ID NO:1, comprenant les étapes de transformation d'un micro-organisme avec un vecteur d'expression contenant une séquence nucléotidique codant ladite protéine P64k ou ladite protéine de fusion, de culture du micro-organisme transformé pour obtenir l'expression de ladite protéine P64k ou de ladite protéine de fusion, et d'isolation dudit produit d'expression.

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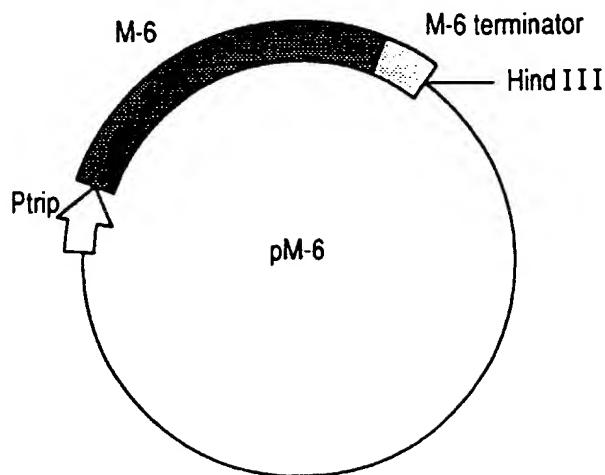


FIG. 1

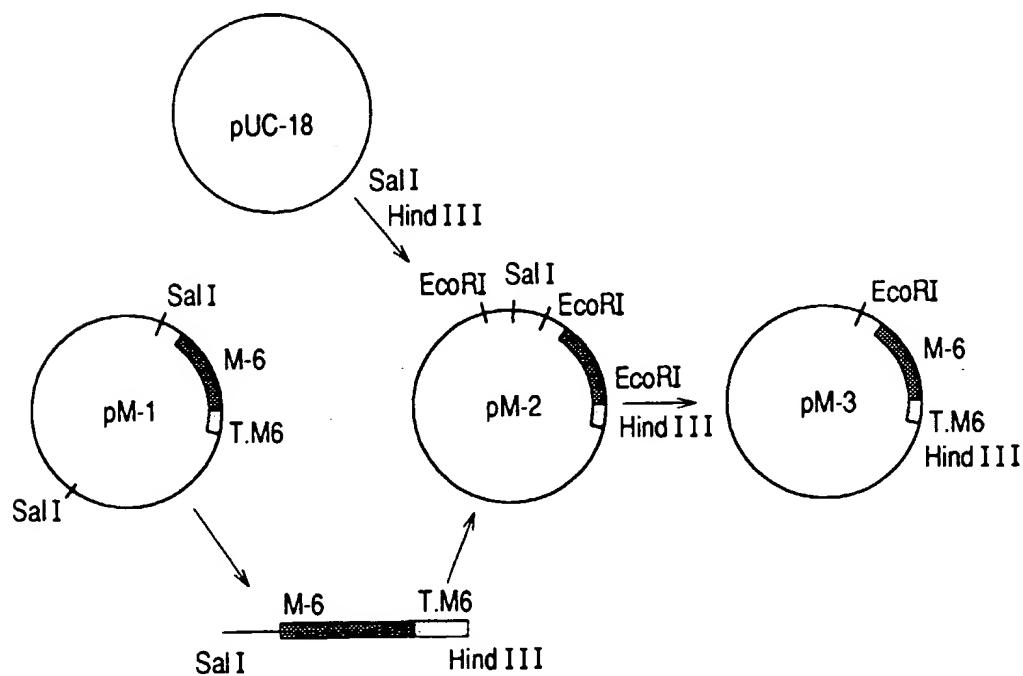
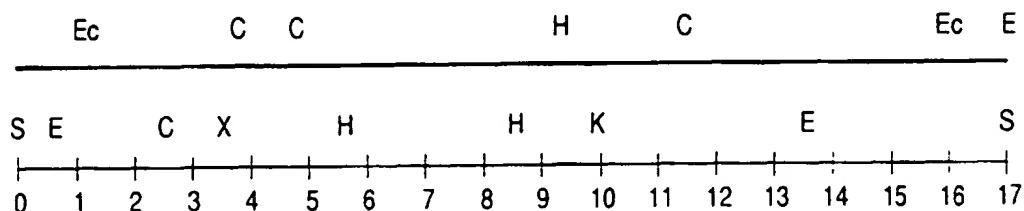


FIG. 2

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S. Sal I; E. EcoRV; Ec. EcoRI; C. ClaI;  
X. XhoI; H. HindIII; K. KpnI.

FIG. 3

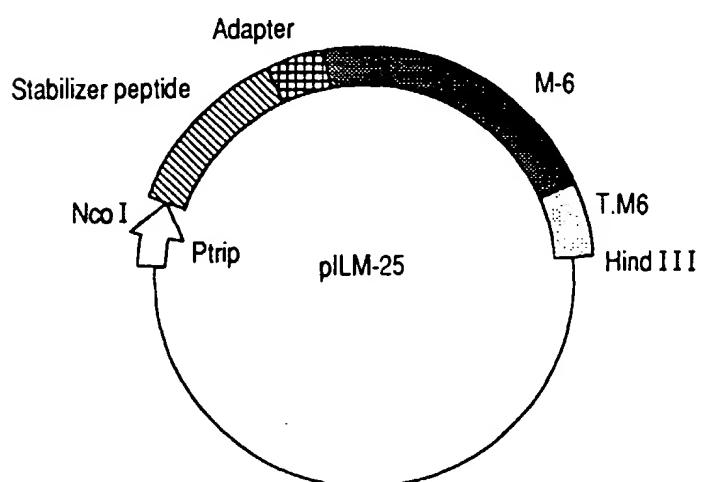


FIG. 4

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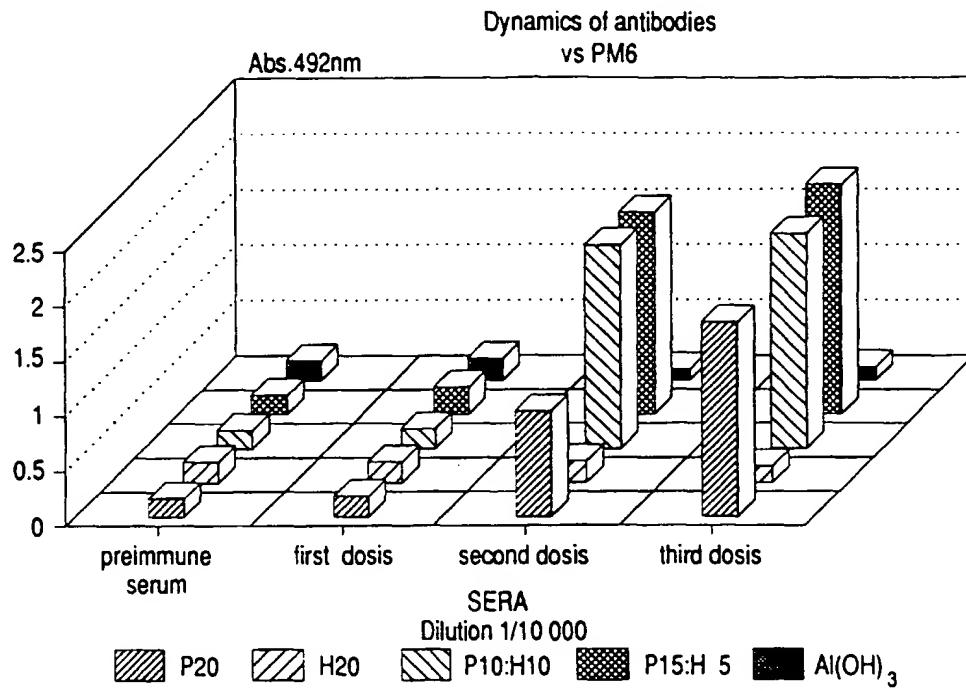


FIG. 5

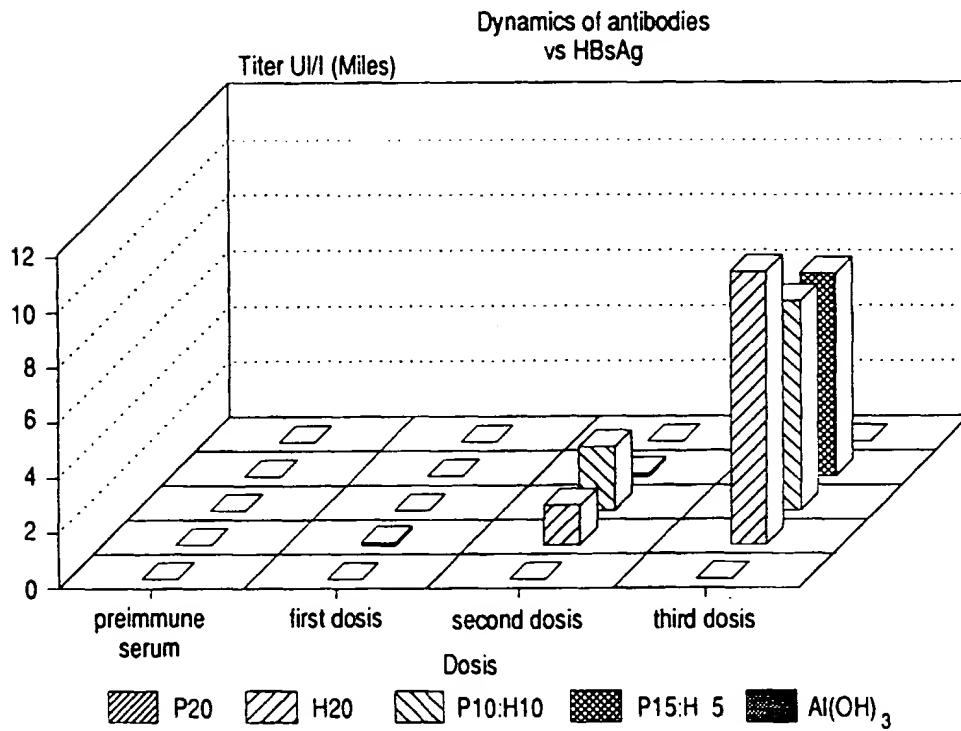


FIG. 6